

THE USE OF PDE4D IN THE SCREENING FOR MEDICAMENTS AGAINST ATHEROSCLEROSIS

PDE4D in atherosclerosis

The PDE4 phosphodiesterases exist as a family of four enzymes, PDE4A, PDE4B, PDE4C and PDE4D. The PDE4 isoenzymes specifically degrade cAMP and are a common target for such pharmacological agents as antidepressants (for example, rolipram). Of the PDE4D isoform, several splice forms are known. Among them are the
5 long isoforms, of which 6 are known, namely PDE4D3, PDE4D4, PDE4D5, PDE4D6, PDE4D7 and PDE4D8. All of these have in common the LR1 and UCR1 sites and the domains located at the C-terminus of these sites, but they have different N-terminal domains. Isoform PDE4D5 was disclosed by Bolger et al. (Characterization of five
10 different proteins produced by alternatively spliced mRNAs from the human cAMP-specific phosphodiesterase PDE4D gene, Biochem. J.(1997), 328, 539-548). Isoform PDE4D7 was recently disclosed in WO02/074992. The PDE4D gene locus has been linked to stroke (WO02/074992). However, there has been no indication so far for an involvement of PDE4D in atherosclerosis or restenosis.

15 In the present invention PDE4, preferably PDE4D, more preferably PDE4D5 or PDE4D7, was identified as a novel target for the identification of compounds that can be used for the treatment of atherosclerosis, preferably of Peripheral Arterial Occlusive Disease (PAOD), or for the treatment of restenosis.

20 In the present invention, PDE4 was identified as a novel target for identifying compounds for therapy of atherosclerosis, preferably of Peripheral Arterial Occlusive Disease (PAOD), or of restenosis. As shown in Figures 7 and 8, the PDE4 inhibitor cilomilast improved walking capacity in a rat model for PAOD. In a preferred
25 embodiment, the novel target is PDE4D, in an even more preferred embodiment, the novel target is PDE4D5 (Seq ID No. 4 and the homologues from other species) or PDE4D7 (Seq ID No. 1 to 3 and homologues from other species). In a most preferred embodiment, the novel target is PDE4D7. As shown in Figures 1 and 4, PDE4D5 and

especially PDE4D7 are up-regulated in the media and intima of balloon-injured rat carotid arteries.

Thus, the present invention provides a novel use of PDE4, preferably PDE4D, more preferably of PDE4D5 or PDE4D7, for identifying a compound which inhibits atherosclerosis, preferably Peripheral Arterial Occlusive Disease (PAOD), or restenosis. Most preferably, PDE4D7 is used.

The present invention also provides a novel process for identifying and obtaining a compound for therapy of atherosclerosis, said process comprising measuring the activation or inhibition of the phosphodiesterase activity of PDE4, preferably PDE4D, more preferably of PDE4D5 or PDE4D7, most preferably PDE4D7, and a compound identified by said process. Most preferably, said compound is an inhibitor of PDE4, preferably PDE4D, more preferably of PDE4D5 or PDE4D7. Most preferably, said compound is an inhibitor of PDE4D7. Procedures to measure phosphodiesterase activity are well known in the art. One non-limiting example for such an assay is described in the examples. The identification of compounds for therapy of atherosclerosis, preferably of Peripheral Arterial Occlusive Disease, or of restenosis may also involve administration of compounds suspected to inhibit PDE4, preferably PDE4D, more preferably PDE4D5 or PDE4D7, most preferably PDE4D7, to an animal in which atherosclerosis, preferably Peripheral Arterial Occlusive Disease, or restenosis is induced, such as in the rat balloon-injury model, or, as another non-limiting example, in ApoE knockout mice which are fed a Western Type diet or a normal Chow diet as a control (eg described by Nakashima et al., ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree, *Arterioscler. Thromb.* (1994) Jan;14(1):133-40) or in the lauric acid model (Kawamura et al., 1985, *Arzneimittelforschung* 35/7A):1154-1156). Preferably, said animal is a non-human animal. Thus, the present invention also provides a process for identifying and obtaining a compound for therapy of atherosclerosis, preferably of Peripheral Arterial Occlusive Disease, or restenosis, said process comprising administering a compound suspected to be an activator or inhibitor of PDE4, preferably PDE4D, more preferably of PDE4D5 or PDE4D7, to an animal in which atherosclerosis, preferably Peripheral Arterial Occlusive Disease, or restenosis is induced, and measuring the extent of atherosclerosis, preferably of Peripheral Occlusive Disease, or restenosis as compared to placebo or carrier-treated animals. Most preferably, said compound is an

inhibitor of PDE4, preferably PDE4D, more preferably of PDE4D5 or PDE4D7, most preferably of PDE4D7.

A process for identifying activators or inhibitors for PDE4D may comprise using a core PDE4D construct (Seq ID No. 5) which is a PDE4D with an amino acid sequence
5 common to all PDE4D long form isoforms. Figure 5 shows an inhibition of core PDE4D activity by Rolipram.

As used herein, the terms „activator or inhibitor of PDE4“, „activator or inhibitor of PDE4D“, „activator or inhibitor of PDE4D5 or PDE4D7“ refer to compounds that activate or inhibit PDE4, PDE4D, PDE4D5 and/or PDE4D7 cellular function, either by
10 acting directly on the phosphodiesterase of PDE4, PDE4D, PDE4D5 and/or PDE4D7, or by modulating indirectly the function of PDE4, PDE4D, PDE4D5 and/or PDE4D7, eg. by altering its subcellular targeting.

The present invention also pertains to a compound identified by any of the
15 processes hereinbefore described.

Further to this, the present invention pertains to a pharmaceutical composition comprising an activator or inhibitor of the phosphodiesterase activity of PDE4, preferably PDE4D, more preferably of PDE4D5 or PDE4D7, identified by the process
20 herein before described, and a pharmaceutically acceptable carrier. Most preferably, said pharmaceutical composition comprises an inhibitor of the phosphodiesterase activity of PDE4, preferably PDE4D, more preferably of PDE4D5 or PDE4D7.

The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of
25 sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

As used herein, "pharmaceutically acceptable salts" refer to derivatives of the identified agents wherein the parent agent is modified by making acid or base salts
30 thereof. Examples of pharmaceutically acceptable salts include, but are not limited to, mineral or organic acid salts of basic residues such as amines; alkali or organic salts of acidic residues such as carboxylic acids; and the like. The pharmaceutically acceptable

salts include the conventional non-toxic salts or the quaternary ammonium salts of the parent compound formed, for example, from non-toxic inorganic or organic acids. For example, such conventional non-toxic salts include those derived from inorganic acids such as hydrochloric, hydrobromic, sulfuric, sulfamic, phosphoric, nitric and the like; and the salts prepared from organic acids such as acetic, propionic, succinic, glycolic, stearic, lactic, malic, tartaric, citric, ascorbic, palmoic, maleic, hydroxymaleic, phenylacetic, glutamic, benzoic, salicylic, sulfanilic, 2-acetoxybenzoic, fumaric, benzenesulfonic, toluenesulfonic, methanesulfonic, ethane disulfonic, oxalic, isethionic, and the like.

The pharmaceutically acceptable salts of the present invention can be synthesized from the parent agent which contains a basic or acidic moiety by conventional chemical methods. Generally, such salts can be prepared by reacting the free acid or base forms of these compounds with a stoichiometric amount of the appropriate base or acid in water or in an organic solvent, or in a mixture of the two; generally, nonaqueous media like ether, ethyl acetate, ethanol, isopropanol, or acetonitrile are preferred. Lists of suitable salts are found in Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Company, Easton, PA, 1985, p. 1418, the disclosure of which is hereby incorporated by reference.

The agents identified by the method of the invention may be modified to achieve (i) modified site of action, spectrum of activity, and/or (ii) improved potency, and/or (iii) decreased toxicity (improved therapeutic index), and/or (iv) decreased side effects, and/or (v) modified onset of action, duration of effect, and/or (vi) modified kinetic parameters (resorption, distribution, metabolism and excretion), and/or (vii) modified physico-chemical parameters (solubility, hygroscopicity, color, taste, odor, stability, state), and/or (viii) improved general specificity, organ/tissue specificity, and/or (ix) optimized application form and route by (i) esterification of carboxyl groups, or (ii) esterification of hydroxyl groups with carbon acids, or (iii) esterification of hydroxyl groups to, e.g. phosphates, pyrophosphates or sulfates or hemi succinates, or (iv) formation of pharmaceutically acceptable salts, or (v) formation of pharmaceutically acceptable complexes, or (vi) synthesis of pharmacologically active polymers, or (vii) introduction of hydrophilic moieties, or (viii) introduction/exchange of substituents on aromates or side chains, change of substituent pattern, or (ix) modification by introduction of isosteric or bioisosteric moieties, or (x) synthesis of homologous compounds, or (xi) introduction of branched side chains, or (xii) conversion of alkyl substituents to cyclic analogues, or (xiii) derivatisation of hydroxyl group to ketals, acetals, or (xiv) N-acetylation to amides, phenylcarbamates, or (xv) synthesis of Mannich bases, imines, or (xvi) transformation of ketones or aldehydes to Schiff's bases,

oximes, acetals, ketals, enolesters, oxazolidines, thiozolidines or combinations thereof; and (b) formulating the product of said modification with a pharmaceutically acceptable carrier or a carrier/diluent acceptable for fragrance or flavor compositions or products.

Any conventional carrier material can be utilized. The carrier material can be an organic or inorganic one suitable for eteral, percutaneous or parenteral administration. Suitable carriers include water, gelatin, gum arabic, lactose, starch, magnesium stearate, talc, vegetable oils, polyalkylene-glycols, petroleum jelly and the like. Furthermore, the pharmaceutical preparations may contain other pharmaceutically active agents. Additional additives such as flavoring agents, stabilizers, emulsifying agents, buffers and the like may be added in accordance with accepted practices of pharmaceutical compounding.

In one embodiment, a method of the present invention involves the administration of a therapeutically effective amount of an antisense oligonucleotide having a sequence capable of binding specifically with any sequences of genomic DNA or an mRNA molecule which encodes a PDE4, preferably PDE4D, or more preferably PDE4D5 or PDE4D7, so as to prevent transcription or translation of PDE4 mRNA, preferably PDE4D, more preferably PDE4D5 or PDE4D7 mRNA, most preferably PDE4D7 mRNA. By "antisense" is meant a composition containing a nucleic acid sequence which is complementary to the "sense" strand of a specific nucleic acid sequence. Once introduced into a cell, the complementary nucleotides combine with endogenous sequences produced by the cell to form duplexes and to block either transcription or translation. See, e.g., Agrawal, S., ed. (1996) Antisense Therapeutics, Humana Press Inc., Totawa NJ; Alama et al. (1997) Pharmacol. Res. 36:171-178; Crooke, S.T. (1997) Adv. Pharmacol. 40:1-49; and Lavrosky et al. (1997) Biochem. Mol. Med. 62(1):11-22. Antisense sequences can be any nucleic acid material, including DNA, RNA, or any nucleic acid mimics or analogs. See, e.g., Rossi et al. (1991) Antisense Res. Dev. 1:285-288; Pardridge et al. (1995) Proc. Nat. Acad. Sci. 92:5592-5596; Nielsen and Haaima (1997) Chem. Soc. Rev. 96:73-78; and Lee et al. (1998) Biochemistry 37:900-1010. Delivery of antisense sequences can be accomplished in a variety of ways, such as through intracellular delivery using a recombinant vector.

Antisense oligonucleotides of about 15 to 25 nucleic acid bases are typically preferred as such are easily synthesized and are capable of producing the desired inhibitory effect. Molecular analogs of antisense oligonucleotides may also be used for this purpose and can have added advantages such as stability, distribution, or limited

toxicity advantageous in a pharmaceutical product. In addition, chemically reactive groups, such as iron-linked ethylenediamine-tetraacetic acid (EDTA-Fe), can be attached to antisense oligonucleotides, causing cleavage of the RNA at the site of hybridization. These and other uses of antisense methods to inhibit the *in vitro* translation of genes are well known in the art. See, e.g., Marcus-Sakura (1988) Anal. Biochem. 172:289.

Inhibition of PDE4, preferably PDE4D, more preferably PDE4D5 or PDE4D7, most preferably PDE4D7 may also be achieved by using RNA interference. RNA interference may be obtained, as a non-limiting example, by the process disclosed in GB 2372995 for inhibiting the expression of a target gene in cells or tissue comprises infection of said cells or tissue with (a) viral particles containing single stranded ribonucleic acid (ss RNA) expressing a sense RNA strand and (b) viral particles containing single stranded ribonucleic acid (ss RNA) expressing an anti-sense RNA strand, wherein the sense and anti-sense RNA strands comprise homologous nucleotide sequences to a portion of said target gene.

15

Rat carotid artery balloon injury is a well accepted technology to study the proliferative events in arteries. It was originally used to help the analysis of the smooth muscle cell proliferative component of atherosclerosis, but recently was also used as a model of restenosis after angioplasty. The SMC response to injury is similar in femoral and carotid arteries. Since it is technically much easier and experimentally much more reproducible the model was applied to the carotid artery. By all means it is intended to serve as model of a hall mark of Peripheral Arterial Occlusive Disease PAOD which is atherosclerosis in femoral arteries. As shown in Table 1, the PDE4 inhibitor Rolipram inhibits neointima formation in this model.

25

Thus, the present invention also provides a method of treatment of atherosclerosis, preferably of Peripheral Arterial Occlusive Disease, or of restenosis comprising administering an activator or inhibitor of PDE4, preferably PDE4D, more preferably of PDE4D5 or PDE4D7 to a subject suffering of atherosclerosis, preferably Peripheral Arterial Occlusive Disease, or restenosis. In a most preferred embodiment, an inhibitor of PDE4, preferably PDE4D, more preferably of PDE4D5 or PDE4D7, most preferably of PDE4D7 is administered. Thus, the present invention pertains to the use of an activator or inhibitor of PDE4, preferably PDE4D, more preferably PDE4D5 or

30

PDE4D7 for the preparation of a medicament for the treatment of atherosclerosis, restenosis or, preferably, Peripheral Arterial Occlusive Disease. In a most preferred embodiment, an inhibitor of PDE4, preferably PDE4D, more preferably of PDE4D5 or PDE4D7, most preferably of PDE4D7, is used.

5

The present invention also provides the compounds, processes, uses and compositions substantially as hereinbefore described, especially with reference to the foregoing examples.

10

Brief description of the figures:

Figure 1:

(a) The catheter injured carotid samples are shown on top. Clearly, a ca. 80 kDa protein is detected with the affinity-purified anti-PDE4D7 rabbit polyclonal antibody. PDE4D7 was neither detected in the non-injured right carotid ((a) bottom) nor in the control carotids from untreated animals (b), indicating a strong induction of PDE4D7 expression as a consequence of balloon catheter injury.

20 **Figure 2:**

A: Cross-reactivity human and rat PDE4D5 N-terminus: human and rat PDE4D5 are 98.85% identical.

B: The comparisons between UCR1, UCR2 and the catalytic domains of the PDE4 subfamilies A, B, C and D show that the UCRs are about as well-conserved as the catalytic domain.

25

Figure 3:

Cross reactivity human-rat-mouse PDE4D7:

Q8CG05: mouse PDE4D7 (Seq ID No. 1); Q8CH04: rat PDE4D7 (Seq ID No. 2);

Q8IVD2: human PDE4D7 (Seq ID No. 3)

human-rat: 96.8 %

5 rat-mouse: 98.8 %

Figure 4:

PDE4D5 and PDE4D7 expression in balloon-injured rat carotids

Lanes 1,2,3: Media and intima, balloon-injured, left carotid, 3 days post balloon injury

10 Lanes 4,5,6: Media, right carotis (uninjured, control), 3 days

Lane 7: media and intima, balloon-injured, left carotid, 14 d post injury

Lane 8: right carotid, non-injured, control, 14 days

Lanes 9,10: media and intima, balloon-injured, left carotid, 7 days post injury

Lanes 11,12: right carotid, non-injured, 7 days

15

Figure 5:

Rolipram inhibition. Phosphodiesterase activity of the PDE4D core construct (DC) can be inhibited by Rolipram. The IC₅₀ of DC phosphodiesterase activity by Rolipram inhibition was 0.34+/- 0.06 μ M.

20

Figure 6:

Effect of PDE4 inhibitor Cilomilast on Walking Capacity in the Lauric Acid model: Animals were selected according to their ability to walk on the treadmill and matched in 4 groups. The PDE3 inhibitor Cilostazol was used as a positive control.

Figure 7:

Effect of PDE4 inhibitors on Walking Capacity in the Lauric Acid model: Maximal walking distance was measured in weekly intervals.

5

Figure 8:

Individual results of the effects of PDE4 inhibitor on Walking Capacity in the Lauric Acid rat model.

10 **Figure 9:**

Effect of PDE4 inhibitors on Body Weight in the Lauric Acid rat model.

Figure 10:

Side effects of PDE4 inhibitor in the lauric acid rat model: Abnormal Stool/Diarrhea.

15

Figure 11:

PK analysis of lauric acid experiment: Plasmalevels of PDE4 inhibitor after oral dosing.

Examples20 **PDE4D5 and PDE4D7 expression in injured rat carotid arteries****Animal surgery**

Male (300 - 400g) Wistar Kyoto Rats (RoRo) were obtained from BRL
CH-Füllinsdorf. The animals were anaesthetized with 5mg/kg Xylazine (Rhompun,
25 Bayer, FRG) and 50mg/kg Ketamin (Ketasol 100, Graeb, CH) i.p. The left carotid was

exposed at the bifurcation and a 2F embolectomy catheter (Edwards laboratories, USA) was inserted. The inflated balloon was pulled through the common carotid artery three times. After permanent ligation of the external carotid artery the wound was closed and the animals kept in pairs with commercial chow and water ad libidum.

5

Tissue harvest

6 weeks post injury the animals were reanesthetized and killed with an i.v. overdose of anesthetics. After opening the body cavity, rats are perfused with cold PBS via a catheter placed in the aortic arch to flush out the blood, and the carotid arteries
10 harvested. The adventitial issues were removed from the arteries with watchmaker forceps. The carotids were opened longitudinally and any remaining endothelium was removed by sliding movements of the forceps. The carotids consist now only of smooth muscle cell tissue. At this stage the carotids were shock frozen in liquid nitrogen, pooled and stored at -80 degrees Celsius.

15

Experimental groups

4 experimental groups were pooled:

1. 11 balloon injured carotids 6 weeks post ballooning
2. 11 contralateral uninjured carotid arteries
- 20 3. 12 left carotids of unmanipulated rats
4. 12 contralateral right carotids form unmanipulated rats

Antibodies

An anti-peptide antibody was generated based on the specific sequence in human
25 PDE4D7 (H₂N-CADLKSESENIQRPTS-CONH₂). This antibody was purified using the column-bound synthetic peptide for affinity chromatography. The specificity of this antibody was tested by Western blotting using recombinant preparations of human PDE 4D3, PDE4D5, PDE4D6, PDE4D7, PDE4D8 as samples. The antibody exclusively

detected hPDE4D7 in these experiments. The ability of the antibody to cross-react with rat or mouse is suggested by the high degree of conservation between human, rat or mouse PDE4D7, as shown in Figure 3.

5 An anti-PDE4D5 peptide antibody was prepared and characterized in a similar way based on the specific sequence H₂N -CEKSKTARKSVSPKLSP- CONH₂. Again, the ability to cross-react is suggested by the high degree of identity in the N-terminal portion of human and rat PDE4D5, as shown in Figure 2.

Preparation of samples for Two-Dimensional Electrophoresis

10 The frozen carotid arteries were powdered in a mortar with liquid nitrogen cooling. The homogenate was taken up in sample solution (7 M urea, 2 M thiourea, 50 mM Tris, 2% (w/v) CHAPS (2-[(3-Cholamidopropyl)dimethyl-ammonio]1-propane sulfonate, Roche Diagnostics, Mannheim, Germany), 0.4% (w/v), Dithioerythritol, 0.5% (v/v) ampholytes (Resolytes 3.5 – 10, BDH, Poole, England)) and left at room
15 temperature for 15 min. The homogenate was centrifuged at 100,000 x g for 1 h at 4°C and the supernatant was collected. The protein concentration was estimated using the BioRad protein assay.

Two-Dimensional Polyacrylamide Gel Electrophoresis

20 Immobilized pH gradient strips (11cm, pH 4 – 7, Amersham Biosciences, Little Chalfont, England) were re-swollen in 7 M urea, 2 M thiourea, CHAPS, 0.4% (w/v), Dithioerythritol, 0.5% (v/v) ampholytes for 6 h, and placed into a Protean IEF cell cup loading tray (BioRad, Hercules, CA). Equal protein amounts (0.5 mg) of the samples were loaded into the cups and isoelectric focusing was performed using the following
25 protocol: 250 V, 2h; gradual increase to 2500 V over 8 h; 2500 V for 8 h. The strips were equilibrated by two consecutive incubations in 6 M urea, 50 mM Tris-HCl, pH 7.5, 30% (v/v) glycerol, 2% (w/v) SDS, 30 mM Dithioerythritol, and in 6 M urea, 50 mM Tris-HCl, pH 7.5, 30% (v/v) glycerol, 2% (w/v) SDS, 136 mM Thioacetamide for 15 min each. The equilibrated strips were placed into the IEF well of a Criterion 4-15% gels. SDS-
30 polyacrylamide electrophoresis and blotting to nitrocellulose membranes (BioRad) was performed according to the gel manufacturer's recommendations. A molecular weight marker (Magic Marker, Invitrogen) was included for molecular weight estimation.

Western Blotting

The blots were blocked with 5% non-fat dry milk in TBS with shaking overnight at 4°C. After washing, 100 ng/ml of the affinity-purified anti-PDE4D7 antibody in TBS + 0.1% (v/v) Tween 20 was added and the blots were incubated with shaking for 90 min at room temperature. The blots were washed and peroxidase-conjugated anti-rabbit antibody was added (dilution 1/50000, BioRad) and incubated with the blots for another 90 min. After washing, the blots were developed with Super Signal West Femto substrate (PIERCE, Rockford, IL) and exposed to film for 5 – 10 min.

10

Inhibition of neointima formation in balloon catheter injured rat carotid arteries by the PDE4 inhibitor rolipram

Drug application

Rolipram at appropriate concentration was prepared in PEG400 (25mg or 2.5mg/ml) and loaded into osmotic minipumps (2002 Alzet) to deliver a constant dose of 0.8 or 8mg/kg/d respectively per rat. The minipump was placed sc. in the neck position of the rat under anesthesia during surgery for the balloon catheter injury. The minipump was connected to the jugular vein via a sylastic catheter to ensure constant i.v. infusion over the entire experimental period of 14d.

20

Plasma levels were determined at the end of the experiment using LCMSMS.

Results following balloon catheter injury (see under animal surgery above):

As shown in Table 1, rolipram inhibited neointima formation in balloon catheter injured rat carotid arteries significantly at 0.8 and 8mg/kg/day iv. To confirm the results, the higher dose was repeated in an independent experiment with a new set of rats (exp 2). At the end of the experiment still 20% of the original volume is present in the pumps due to the slower pump rate of a PEG 400 solution rather than water. Thus, the plasma levels determined at the end of the experiment reflect steady state exposure. It is interesting to

note that plasma levels are well within the expected range of K_i of Rolipram for PDE4 enzymes.

Experiment	Rolipram dose	Inhibition of neointima	plasma level	p	n
(number)	(mg/kg/d)	(% from placebo)	(nM)		
2002-33	8	48 570 ± 70	>0.05	10	
2002-33	0.8	33 70 ± 24	>0.05	10	
2003-2	8	37 420 ± 150	>0.05	9	

10

Table 1 shows the summary of the efficacy data of rolipram mediated inhibition of neointima formation as measured by histomorphometry on plastic embedded cross sections (2 per carotid).

15 Expression of recombinant PDE4D5 and PDE4D7 isoforms

Cloning of the PDE4D isoforms 5 and 7 and the core construct

Core construct:

A cDNA encoding the core fragment that is common for all the PDE4D isoforms 3-
 20 8 starting with the amino acid sequence FDV carboxyterminal to the LF1 splice site was generated by PCR using a 5' oligonucleotide with a HindIII cloning site (gatgaattcaagcttttgatgtggacaatggcaca) introducing two additional amino acid (K and L) in front of the FDV sequence. At the 3' end a set of primers was used that either generated the native sequence (gtgatatctcattatcacgtgtcaggagaacgatcatctatgaca) or added a
 25 sequence encoding 6xHis residues (gtgatatctcattatcaatgggatggtgatggtgcgtgtcaggagaacgatcatctatgac) to enable rapid purification of the recombinant proteins. The cDNA encoding the core construct was cloned as a EcoRI-EcoRV fragment into the expression vector pENTRTM1a (GIBCO/BRL)

Isoforms (except core construct):

The DNA fragments encoding the isoform specific N-termini were generated by using synthetic oligonucleotides with terminal restriction enzyme sites for EcoRI and HindIII incorporated for directional cloning. These isoform specific DNA fragments
5 were fused to the core construct sequence via the HindIII site introducing two additional amino acid residues (K and L). The integrity of the clones was confirmed by DNA sequencing prior to expression.

Expression of the PDE4D isoforms and the core construct in insect cells

10 The cDNAs were cloned into the pFASTBAC1 vector (Life Technologies, Inc) for expression in insect cells and the products were confirmed by sequencing. After recombination into the baculovirus genome the purified viral DNAs were transformed into the insect cells. Sf9 cells were cultured at 27°C in TC100 medium (BioWhittaker) with 5% (v/v) fetal calf serum. Virus stocks were generated with a titer of 1.5×10^9 pfu/ml.
15 For large scale production of the isoforms 1-24 L fermentations of Sf9 cells were infected with a MOI of 1.

In one example, the 6xHis tagged PDE4D polypeptides DC, D5 and D7 were produced in Sf9 cells in 1L spinner flasks using SF1 medium in the absence of serum.
20 Infected cells were harvested 3 days after infection with the recombinant baculoviruses.

In another example, the PDE4D core construct DC was produced in a 24 L Airlifter Fermenter with 15 L medium (SF1 with 0% serum), 0.15 L lipids and 9 L Sf9 cells. During the entire fermentation procedure the cells were cultured at pH 6.2, 27.0 \pm 0.2°C
25 and a pO₂ of 30.0 \pm 0.5 %. Cells were grown for 3 days. The cell number at infection was 2.3×10^6 cells/ml. Cells were infected with 450 ml recombinant baculovirus. Cells were harvested at 68h post infection and the cell pellet as well as the concentrated supernatant stored at -80°C until further processing.

30 Purification of 6xHis tagged PDE4D isoforms

Sf9 cells from 1 liter culture broth, overexpressing the respective isoform, were resuspended on ice in 50 ml 50 mM HEPES pH 7.8, 300 mM NaCl 10 mM imidazole, 1 mM DTT, supplemented with protease inhibitors (one protease inhibitor cocktail tablet "complete, EDTA-free"; Roche). Opening of cells was performed by use of a 50 ml
5 Dounce homogenizator and the homogeneous mixture was centrifuged for 1 hour at 70'000 g and 4°C (Kontron TFT 45.94 rotor at 30'000 rpm). The supernate was filtrated through a filter with a pore size of 1.2 µM (Minisart; Sartorius, Germany) and then applied to a 6 ml Ni-NTA agarose column at 2 ml/min. After equilibration with 50 mM HEPES pH 7.8, 300 mM NaCl, 10 mM imidazole, protein was eluted with a linear 30 ml
10 gradient from 10 to 230 mM imidazole in the same buffer. Fractions containing the PDE4D isoform as analyzed by Coomassie stained SDS-PAGE were pooled and stored frozen at -80°C. Fresh Ni-NTA agarose material was used for every different PDE4D isoform preparation in order to prevent cross contamination of isoforms.

15 Specific activities of 6xHis tagged PDE4D isoforms

Relative concentrations of Ni-NTA agarose purified isoform preparations were estimated by SDS-PAGE. Equal volume amounts of isoform preparations were applied to a gradient gel (4-12% NuPage; Invitrogen). After electrophoresis the Coomassie stained polyacrylamide gel was imaged by a video imaging system. Optical densities of PDE4D
20 bands were integrated using a Macintosh computer and the public domain software "NIH Image", version 1.61 (developed at the U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). The integrated arbitrary units per PDE4D band as returned by the software reflect the relative PDE4D concentrations within the original pools. Identities of PDE4D and tubulin bands had
25 been verified by independent SDS-PAGE, excision of corresponding bands, trypsin cleavage and identification of tryptic peptides by MALDI-MS.

Activities of equal volume amounts of 10⁶-fold diluted purified isoforms were determined by use of a commercial radioactive phosphodiesterase assay (cAMP-dependent phosphodiesterase [³H] assay; Amersham Pharmacia Biotech), following the
30 instructions of the manufacturer. The obtained arbitrary activity units reflect the relative PDE4D activities within the original pools.

Relative specific activities of PDE4D isoforms were calculated by dividing relative activity values by relative concentration values.

Qualitative investigation of aggregation by size exclusion chromatography (SEC)

50 µl of Ni-NTA agarose purified isoform preparation was injected into a Superose 12 size exclusion column (type PC3.2/30; Amersham Pharmacia Biotech), equilibrated with 50 mM TrisHCl pH 7.7, 100 mM NaCl, 0.5 mM MgCl₂ at a flow rate of 0.1 ml/min at 4°C. Chromatograms were recorded at 278 nm. Starting from the elution volume, the column eluate was collected as 50 µl fractions.

Activity assay and inhibition of phosphodiesterase activity

An IMAP FP-Assay was used for the determination of phosphodiesterase activity. The phosphodiesterase activity of the core construct and PDE4D3, 5 or 7 was measured using the HEFP Phosphodiesterase Assay Kit (Molecular Devices). 2 µl of PDE4D5 or 7 or PDE4D core construct, 2 µl of cAMP (to a final concentration of 40 nM) and 1 µl of test substance or carrier were incubated for 45 min on a shaker. 12 µl of Binding Solution provided by the kit (with beads diluted 1:320) were added, and the reaction mixture incubated on a shaker for 2 hours. Fluorescence polarisation of the samples was measured in a Packard BioScience Fusion a-FP HT using as an emission filter a Polarizer 535, and as an excitation filter, Fluorescein 485/20. Inhibition of the phosphodiesterase activity of the PDE4D core construct was determined using Rolipram as inhibitor, and using PDE4D core construct at 30 ng/ml.

Rat Lauric Acid model for PAOD (Kawamura et al., 1985, Arzneimittelforschung 35/7A):1154-1156)

Rats were injected intra-arterially with 75µg of Lauric acid in 10µl of pure ethanol. The injection site at the proximal femoral artery was closed with histo-acryl. The wound was closed and the animal allowed to recover. Rats had been trained to walk on a treadmill prior to LA injection as indicated in Fig 6. Drugs were applied by gavage as indicated. The treatment causes inflammation in small vessels, which can lead to PAOD. Rats were forced to exercise on the treadmill at 25m/min and 4.5% slope until they fatigued. Fatigue was defined as the time needed until a total of 5 small electric stimuli have been cumulatively applied as re-enforcement for the rat to run on the treadmill.

The PDE4 inhibitor cilomilast was compared to the PDE3 inhibitor Cilostazol. Cilostazol is used in the treatment for PAOD and thus serves as a positive control. Cilomilast showed equipotent efficacy as compared to Cilostazol (Fig. 7).

5

This experiment indicates that a PDE4 inhibitor can improve walking distance in a rat model for PAOD.